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Endotoxin-neutralizing activity of hen egg phosvitin

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ABSTRACT

Endotoxin, also known as lipopolysaccharide (LPS), is responsible for initiating host responses leading to inflammation and sometimes unwanted sepsis, which is associated with high mortality in patients. No therapeutic agents to date are efficacious enough to protect patients from LPS-mediated tissue damage and organ failure. Previously, egg yolk protein phosvitin (Pv) in zebrafish has been shown to act as a pattern recognition receptor, capable of binding to the microbial cell wall components including LPS, we therefore wonder if it has the capacity to block LPS toxicity. In this study we first demonstrated that hen Pv, a naturally occurring protein rich in egg yolk, had antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* under thermal stress, and then showed that Pv was able to bind to LPS, lipoteichoic acid and peptidoglycan as well as the microbes *E. coli and S. aureus*. More importantly, we revealed that Pv significantly inhibited LPS-induced tumor-necrosis factor (TNF)-α release from murine RAW264.7 cells and considerably reduced serum TNF-α level in mice. Additionally, hen Pv could promote the survival rate of the endotoxemia mice. Furthermore, hen Pv displayed no cytotoxicity to murine RAW264.7 macrophages and no hemolytic activity towards human red blood cells. Taken together, these data suggest that Pv is an endotoxin-neutralizing agent with a therapeutic potential in clinical treatment of LPS-induced sepsis.

1. Introduction

Most pathogenic microbes are classified as Gram-negative bacteria. A major constituent of the Gram-negative microbial cell wall is endotoxin, also known as lipopolysaccharide (LPS). LPS is one of the highly conserved pathogen-associated molecular patterns (PAMPs) that are recognized by the toll-like receptor 4 (TLR4) of the host's innate immune system. In circulation, binding of LPS to the TLR4, which is prominently expressed on monocytes and macrophages, provokes host cells to produce a large amount of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, by activating several types of transcription factors including the nuclear factor (NF)-κB (Hack et al., 1997; Zhang and Ghosh, 2000). This TLR-mediated innate immunity is responsible for the host defense against invading pathogenic microbes. However, the over-stimulation of the TLR-mediated immunity upon endotoxemia that results from severe microbial infection can lead to sepsis with resultant multiple organ dysfunction syndrome (MODS), a serious disease which is associated with high mortality in intensive care unit patients (Parrillo, 1993; Warren, 1997).

The therapy of sepsis rests on antibiotics, surgical drainage of infected fluid collections, fluid replacement and appropriate support for organ dysfunction. However, no therapeutic agents to date are efficacious enough to protect patients from LPS-mediated tissue damage and organ failure (Russell, 2006). Despite the use of corticosteroids, broad-spectrum antibiotics (e.g., imipenem) and fluid resuscitation, the mortality rate of patients with severe sepsis is still over 50% (Rice and Bernard, 2005). Therefore, search for novel therapeutics that are able to halt the LPS stimulation on the host immunity remains urgent and pressing.

To block the recognition of LPS by TLR4, both anti-LPS antibody and TLR4 antagonistic peptides have been developed. Their protection on both laboratory animals and patients against MODS has been widely tested, but the trials have not been satisfactory in reducing mortality significantly (Bone et al., 1995; Leaver et al., 2007; Ziegler et al., 1991). Recently, antimicrobial peptides (AMPs) have emerged as ideal candidates for sepsis therapeutics because they do not only have a broad-spectrum activity against microbes but also possess the potential to bind LPS and block LPS-stimulated

Abbreviations: AMPs, antimicrobial peptides; BSA, bovine serum albumin; DIG, digoxigenin; FITC, fluorescein isothiocyanate; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MODS, multiple organ dysfunction syndrome; MTT, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF, nuclear factor; PAMPs, pathogen-associated molecular patterns; PBS, phosphate-buffered saline; PGN, peptidoglycan; PMB, polymyxin B; pNPP, p-nitrophenyl phosphate disodium; Pv, phosvitin; RBCs, red blood cells; TBS, Tris-buffered saline; TLR4, toll-like receptor 4.

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cytokine release (Mookherjee and Hancock, 2007; Rosenfeld et al., 2006). AMPs are crucial humoral components of the innate immunity of virtually all organisms, and defend them from the attack by invading pathogens (Bulet et al., 2004; Hancock, 2001). Our previous study has shown that egg yolk protein phosvitin (Pv) in zebrafish (*Danio rerio*) is not only directly microbicidal but binds the PAMPs such as LPS, lipoteichoic acid (LTA) and peptidoglycan (PGN) as well (Wang et al., 2011). Similarly, hen egg yolk protein Pv has been shown to exhibit an antimicrobial activity against *Escherichia coli* owing to its synergistic effect of metal-chelating ability and surfactant activity under thermal stress (Khan et al., 2000). However, it remains unknown if hen Pv can bind the microbial cell wall components PAMPs. In addition, Pv is a major component of egg yolk, accounting for about 7% of the yolk proteins (ABE et al., 1982; Wallace and Morgan, 1986), and naturally occurs in hen egg, a common source of human food, we thus wonder if hen Pv has LPS-neutralizing activity, and if so, to evaluate if it can be an important future consideration for the treatment of sepsis.

2. Materials and methods

2.1. SDS-PAGE

Hen Pv purchased from Sigma (St. Louis, USA) was dissolved in double distilled water, loaded and electrophoresed on a 12% SDS-PAGE gel to check its purity. After electrophoresis, the gel was stained using the modified staining solution containing 0.05% Coomassie brilliant blue R-250, 0.1 mol/L aluminum nitrate, 25% isopropanol, 10% acetic acid and 1.0% Triton X-100, as described by Hegenauer et al. (1977).

2.2. Preparation of microbes

The Gram-negative bacterium *E. coli* and the Gram-positive bacterium *Staphylococcus aureus* were both incubated in L-broth medium containing 10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl to logarithmic growth phase and harvested by centrifugation at $3000 \times g$ at 4 °C for 15 min. After washing by 10 mmol/L phosphate-buffered saline (PBS; pH 7.4) for three times, the microbial pellets were resuspended in PBS, and used for the following experiments.

2.3. Assays for antimicrobial activity

The antimicrobial assay was carried out according to the method of Khan et al. (2000). Both *E. coli* and *S. aureus* cells were diluted with 10 mmol/L PBS (pH 7.4) and microscopically adjusted to yield a concentration of 10^4 cells/ml by using a hematometer. A total of 100 μl of the microbial cell suspension was mixed with an equal volume of Pv solution in 10 mmol/L PBS (pH 7.4) to give a final Pv concentration of 100 or 500 μg/ml. For control, bovine serum albumin (BSA) was used instead of Pv. The mixtures were incubated in a water bath, with gentle rotation, at 37 °C or 50 °C for desired times. After incubation, the mixtures were immediately put on ice, and cooled to room temperature. Aliquots of 50 μl mixtures were sampled, seeded onto LB agar plates ($n = 3$), and incubated at 37 °C overnight. The number of colonies formed was counted, and the survival percent was calculated.

2.4. Assay for binding of Pv to microbial cells

As Pv was able to inhibit the growth of *E. coli* and *S. aureus* under thermal stress (see below), we therefore tested if Pv could bind *E. coli* and *S. aureus*. Pv, which had not been heated at 50 °C, was labeled with fluorescein isothiocyanate (FITC; Sigma, USA) as described by Li et al. (2008). The purity of FITC-labeled Pv was confirmed by SDS-PAGE. The *F*/*P* ratio, which is defined as the ratio of moles of FITC to moles of protein in the conjugate, was calculated by the equation $F/P = (2.77 \times A_{495})/[A_{280} - (0.35 \times A_{495})]$ from the absorbance readings of the conjugate samples. BSA was also labeled with FITC using the same method. A total of 30 μl of FITC-labeled Pv (200 μg/ml) in 10 mmol/L PBS was mixed with 30 μl of *E. coli* and *S. aureus* suspensions (5×10^7 cells), respectively, and incubated at 4 °C overnight. The microbes were then washed three times with 25 mmol/L Tris–HCl buffer containing 137 mmol/L NaCl and 3 mmol/L KCl (Tris-buffered saline, TBS; pH 7.6), harvested by centrifugation at room temperature at $3000 \times g$ for 15 min, and resuspended in 1 ml of TBS. An aliquot of 10 μl of the microbial suspensions was sampled, and applied to microscope slide. The binding of FITC-labeled Pv to the microbial cells was observed under an Olympus BX51 fluorescence microscope. Microbes treated with FITC-labeled BSA under the same conditions were used as control.

2.5. Assay for binding of Pv to various ligands

As Pv was able to bind *E. coli* and *S. aureus*, we then examined if Pv could interact with the ligands LPS, LTA and PGN. The labeling of Pv with digoxigenin (Roche, Germany) was performed according to the instruction of DIG protein labeling kit (Roche, Germany). Briefly, 1 ml of 1 mg/ml Pv, which had not been heated at 50 °C, in 10 mmol/L PBS (pH 7.4) was mixed with 16 μl of 20 mg/ml DIG-NHS solution dissolved in DMSO and incubated at 25 °C for 2 h. The remaining free DIG-NHS was removed by gel filtration on a Sephadex G-25 column.

The binding of Pv to LPS, LTA and PGN was assayed by the method of Li et al. (2008). LPS from *E. coli*, and LTA and PGN from *S. aureus* (all from Sigma, USA) were individually dissolved in double distilled water, yielding a concentration of 40 μg/ml. Aliquots of 50 μl (2 μg) of each ligand solution were added to each well of a 96-well microplate and air-dried at room temperature overnight. The plate was incubated at 60 °C for 30 min to fix the ligands, and the wells were each blocked with 200 μl of 1 mg/ml BSA in 10 mmol/L PBS (pH 7.4) at 37 °C for 2 h. After washing four times with 200 μl of 10 mmol/L PBS supplemented with 1% Tween-20, an aliquot of 50 μl DIG-labeled Pv solution with different concentrations of DIG-labeled Pv (0, 1.25, 2.5, 5, 10, 15, 25 and 50 μg/ml) was added into the wells. After incubation at room temperature for 3 h, the wells were each washed four times with 200 μl of 10 mmol/L PBS supplemented with 1% Tween-20 and then 100 μl of alkaline phosphatase conjugated anti-digoxigenin Fab fragments (Roche, Germany) diluted 1:1000 with 10 mmol/L PBS (pH 7.4) containing 1 mg/ml dry milk powder was added to each well. The wells were incubated at 37 °C for 2 h, washed as above, and reacted with 75 μl p-nitrophenyl phosphate disodium (pNPP) color development solution (pNPP color development kit; Beijing Hapten and Protein Biomedical Institute) at 37 °C for 20 min. Subsequently, 25 μl of 3 mol/L NaOH was added to each well to terminate the reaction, and the absorbances at 405 nm were monitored under a microplate reader (GENios Plus, Tecan). For control, DIG-labeled BSA at the same concentrations was used instead of Pv.

2.6. Assay for effect of Pv on LPS-induced TNF-α release from RAW264.7 cells

Assay for the effect of Pv on LPS-induced TNF-α release was carried out as described by Weiss et al. (2000) with slight modification. Murine macrophage cell line RAW264.7 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. Immediately before incubation with LPS, medium was removed and the cells